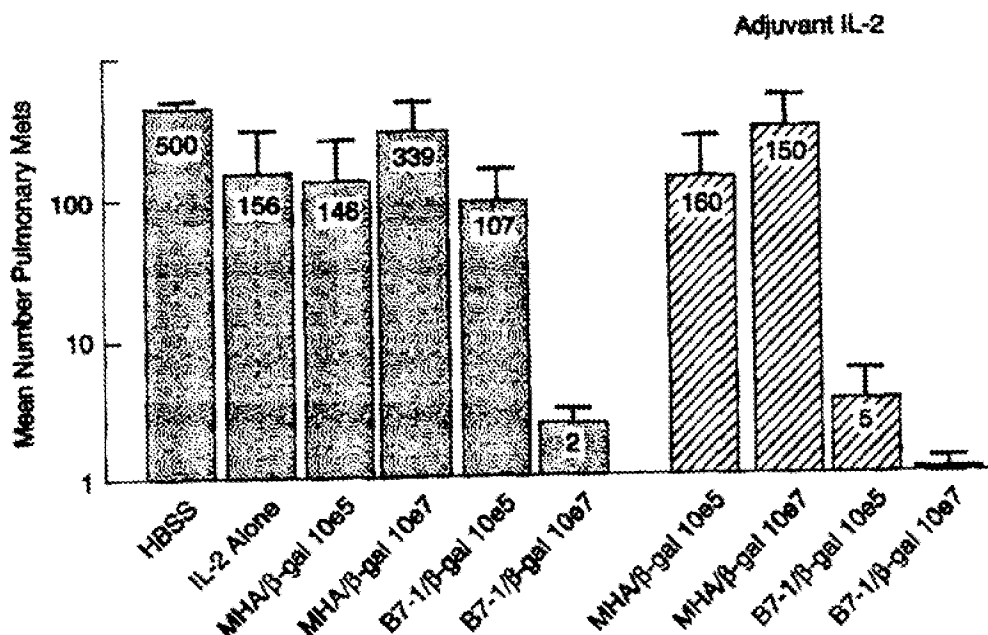




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/86, 15/12, 15/19, C07K 14/705		A2	(11) International Publication Number: WO 96/11279
			(43) International Publication Date: 18 April 1996 (18.04.96)
(21) International Application Number: PCT/US95/12638		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BI, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
(22) International Filing Date: 2 October 1995 (02.10.95)			
(30) Priority Data: 08/317,402 3 October 1994 (03.10.94) US 08/474,639 7 June 1995 (07.06.95) US			
(71) Applicant: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, National Institutes of Health, Suite 325, Box 13, 6011 Executive Boulevard, Rockville, MD 20852 (US).		Published Without international search report and to be republished upon receipt of that report.	
(72) Inventors: ROSENBERG, Steven, A.; 9015 Honeybee Lane, Bethesda, MD 20817 (US). RESTIFO, Nicholas, P.; 1815 18th Street N.W., Unit 302, Washington, DC 20009 (US). MOSS, Bernard; 10301 Dickins Avenue, Bethesda, MD 20814 (US).			
(74) Agents: FEILER, William, S. et al.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).			

(54) Title: ENHANCED IMMUNE RESPONSE BY INTRODUCTION OF CYTOKINE GENE AND/OR COSTIMULATORY MOLECULE B7 GENE IN A RECOMBINANT VIRUS EXPRESSING SYSTEM



(57) Abstract

The present invention is a recombinant virus which has incorporated into its genome or portion thereof a gene encoding an antigen at a disease causing agent in combination with an immunostimulatory molecule for the purpose of stimulating an immune response against the disease causing agent. Methods of treatment of diseases such as cancer and diseases caused by pathogenic microorganisms are provided using the recombinant virus.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

- 1 -

ENHANCED IMMUNE RESPONSE
BY INTRODUCTION OF CYTOKINE GENE AND/OR
COSTIMULATORY MOLECULE B7 GENE IN A
RECOMBINANT VIRUS EXPRESSING SYSTEM

FIELD OF THE INVENTION

5 The present invention relates to recombinant viral
vector vaccines for the prevention or treatment of
pathogenic diseases and cancer. More particularly, it
relates to recombinant viral vector vaccines comprising a
genes encoding an antigens and a gene(s) encoding an
10 immunostimulatory molecule(s).

BACKGROUND OF THE INVENTION

 The five year survival of patients with metastatic
melanoma is less than 2% in most reported series.
Combination chemotherapy can induce objective regressions
15 of melanoma but this treatment is rarely, if ever,
curative. Tumor infiltrating lymphocytes (TIL) have been
identified in patients with melanoma that appear to
recognize unique cancer antigens in an MHC restricted
fashion. (Rosenberg, S.A. J. Clin. Oncol. 10:180-199,
20 1993; Topalian, S.C. et al Tumor-infiltrating lymphocytes:
Evidence for specific immune reactions against growing
cancers in mice and humans. In: De Vita, Hellman and
Rosenberg (eds) Important Advances in Oncology,
Philadelphia, J.B. Lippincott Co., 1990, pp 19-41,
25 Schwartzentruber et al J. Immunol 146:3674-3681, 1991).
The adoptive transfer of TIL can mediate regression in 35
to 40% of patients with advanced melanoma and studies are
underway to attempt to generate more potent TIL by genetic
modification of TIL or by immunization with preparations
30 containing tumor associated antigens. Rosenberg, J. Clin.
Oncol. 10:180-199, 1993; Rosenberg, S.A. J. Am. Med. Assoc
268:2416-2419, 1992.

 A large number of studies in experimental animals
have demonstrated that the cellular rather than the
humoral arm of the immune response plays the major role in
35 the elimination of murine tumors. Wunderlich, J.R. et al.

- 2 -

Principles of tumor immunity: In: DeVita et al (eds)
Biologic Therapy of Cancer, Philadelphia: J.B. Lippincott
Co. 1991, pp 3-21. Much of this evidence was derived from
studies in which the adoptive transfer of T lymphocytes
from immune animals could transfer resistance to tumor
challenge or in some experiments the actual elimination of
established cancer. Thus, most strategies for the
immunization of patients with cancer have been directed at
stimulating strong T cell immune reactions against tumor
associated antigens.

Most attempts at active immunization against cancer
antigens have involved whole tumor cells or tumor cell
fragments, though it would be most desirable to immunize
specifically against unique tumor antigens that
distinguish malignant from normal cells. The molecular
nature of the tumor associated antigens recognized by T
lymphocytes is poorly understood. In contrast to
antibodies that recognize epitopes on intact proteins, T
cells recognize short peptide fragments (8-18 amino acids)
that are presented on cell surface class I or II major
histocompatibility (MHC) molecules and it is likely that
tumor associated antigens are presented and recognized by
T cells in this fashion.

A number of genes have been identified that encode
melanoma tumor antigens recognized by TIL in the context
of the HLA-A2 class I molecule. Kawakami, T. et al Proc.
Nat'l Acad. Sci. 91:3515-3519, 1994; Kawakami, Y. et al J.
Exp. Med. 180:347-352, 1994; Kawakami et al Cancer Res.
54:3124-3126, 1994. These antigens appear to be the most
clinically relevant antigens responsible for mediating
tumor regression in patients with advanced melanoma since
the TIL used to identify these antigens were capable of
mediating in vivo antitumor regression. Two such
antigens, which appear to be present in virtually all
fresh and cultured melanomas, have been called MART-1
(Melanoma Antigen Recognized by T Cells - 1) and gp100.

- 3 -

°

The genes encoding both of the peptides have been cloned and sequenced. The MART-1 gene encodes a 118 amino acid protein of 13 kD. The gp100 gene encodes a protein identical to that recognized by monoclonal antibody HMB-45. With the exception of melanocytes and retina no normal tissues express this antigen and no expression of these gene products has been seen on cancers other than melanoma. Both antigens, therefore, appear to be melanocyte lineage specific.

The MART-1 antigen was expressed on all nine tissue culture lines tested (that were established from melanomas in the Surgery Branch, NCI) and on all fresh melanomas tested. Studies by others showed that the MART-1 (also called Melan-A) antigen was expressed on 26 of 26 fresh melanomas. Coullie, P.G. et al J. Exp. Med. 180:35-42, 1992. The gp100 antigen is also widely expressed in melanomas. In one study, reactivity with antibody HMB-45 (reactive with gp100) was present on 100% of non-spindle cell type melanomas and on 62 of 67 total melanomas. Wick, M.R. et al J. Cutan. Pathol. 4:201-207, 1988. In another study, 32 of 35 melanomas studied (91%) expressed gp100 (Ordonez, N.G. et al Am. J. Clin. Pathol. 4:385-390, 1988) and in a third study of 60 of 62 (97%) melanomas expressed gp100 (Gown, A.M. et al Am. J. Pathol. 123:195-203, 1986).

Of 14 separate TIL cells that were raised in the Surgery Branch, NCI from different HLA-A2 individuals, 13 of 14 recognized MART-1 and 4 of 14 recognized the gp100 antigen. Because TIL cells that recognize these determinants have been shown to be capable of mediating cancer regression in vivo, it appears that these antigens are involved in cancer regression.

Another gene coding for a human tumor specific antigen on a human melanoma was cloned by Van der Bruggen et al (Science 254:1643-1647, 1991). This antigen is coded for by a gene called MAGE-1 which spans five

- 4 -

kilobases. A 2,419 base pair coding sequence produces a predicted protein product of 26 kD. The MAGE antigen is HLA-A1 restricted and the nine amino acid fragment that represents the A1 restricted immunodominant peptide has been defined as Glu-Ala-Asp-Pro-Thr-Gly-His-Ser-Tyr. This nine amino acid peptide is encoded by the third exon of the MAGE gene. Transfection of a 500 base pair fragment of this gene can confer recognition by a MAGE specific CTL clone. Incubation of an EBV cell line with the immunodominant peptide can confer sensitivity to lysis by a MAGE-1 specific CTL clone. MAGE-1 does not appear to be expressed in normal cells with the possible exception of testis, but is expressed on approximately half of metastatic melanomas, about 20% of breast cancers as well as other selected types of cancer.

The identification of an immunodominant peptide that represents a unique tumor antigen has opened new possibilities for immunization against cancer. Substantial evidence exists in animal models that immunization with immunodominant viral peptides can induce viral specific CTL that can confer protection against viral infection. Although pure peptide alone is ineffective in stimulating T cell responses, peptides emulsified in adjuvants or complexed with lipids have been shown to prime mice against challenge with fresh virus and can induce virus specific CTL that protect mice against lethal viral inocula (Kast, W.M. et al Proc. Nat'l Acad. Sci. U.S.A. 88:2283-2287, 1991; Deres, K. et al Nature 342:561-564, 1989; Gao, X.M. et al J. Immunol. 147:3268-3273, 1991; Aichele, P. J. Exp. Med. 171:1815-1820, 1990; Collins, D.S. et al J. Immunol. 148:3336-3341, 1992). Immunization of mice against splenocytes coated with Listeria monocytogenes peptide epitopes also results in the generation of Listeria specific CTL which can be expanded in culture. Adoptive transfer of these CTL can protect mice against lethal bacterial challenge (Harty,

- 5 -

•
J.T. et al J. Exp. Med. 175:1531-1538, 1992). Peptides representing antigenic epitopes of HIV gp120 and gp160 emulsified in complete Freund's adjuvant can also prime specific CTL responses (Takahashi, H. et al Proc. Nat'l Acad. Sci. U.S.A. 85:3105-3109, 1988; Hart, M.K. et al Proc. Nat'l Acad. Sci. U.S.A. 88:9448-9452, 1991).

5
While immunization with peptides in adjuvants or complexed with lipids gives rise to T cell responses in mice, the reactions are rarely strong enough to induce T reactive cells in primary splenocytes. The detection of sensitized lymphocytes almost invariably requires secondary in vitro stimulation.

10
The expression of the B7 gene family has been shown to be an important mechanism of antitumor responses in both mice and humans. It is now becoming apparent that at least two signals are required for activation of naive T-cells by antigen bearing target cells: an antigen specific signal, delivered through the T-cell receptor, and an antigen independent or costimulatory signal leading to lymphokine products (Hellstrom, K.E. et al. Annals NY Acad. Sci. 690:225-230, 1993). Two important costimulatory molecules are B7-1, which is the ligand for T-cell surface antigens CD28 and CTLA4 (Schwartz, R.H. Cell 71:1065-1068, 1992; Chen, L. et al. Cell 71:1093-1102, 1992; Freeman, G.J. et al. J. Immunol 143:2714-2722, 1989; Freeman, G.J. et al. J. Exp. Med. 174:625-631, 1991), and B7-2, an alternative ligand for CTLA4 (Freeman, G.J. et al. Science 262:813-960, 1995). To date, both murine B7-1 and B7-2 (Freeman, G.J. et al. J. Exp. Med. 174:625-631, 1991; Freeman, G.J. et al. Science 262:813-960, 1995) and human B7-1 and B7-2 have been described (Freeman, G.J. et al. J. Immunol 143:2714-2722, 1989; Freeman, G.J. et al. Science 262:909-911, 1993). It is unclear at this time whether the costimulatory signals provided by B7-1 and B7-2 are functionally distinct or redundant mechanisms for T-cell activation (Hathcock, K.S.

15
20
25
30
35

- 6 -

et al. J. Exp. Med. 180:631-640, 1994). Most murine and human tumors do not express B7-1 or B7-2, implying that even when a tumor expresses a potential rejection antigen, it is unlikely to activate antitumor T-cell responses (Hellstrom, K.E. et al Annals. N.Y. Acad. Sci. 690:225-230, 1993); Hellstrom, I. Annals. N.Y. Acad. Sci. 690:24-31, 1993). In essence, anergy may result from only one signal being received by the T-cell (Hellstrom, K.e. et al. Annals. N.Y. Acad. Sci. 690:225-230, 1993).

Transfection of B7 into melanoma cells was found to induce the rejection of a murine melanoma in vivo (Townsend, S.E. et al Science 259:368-370, 1993).

Vaccinia viruses have been extensively used in humans and the use of a vaccina based vaccine against smallpox has led to the worldwide eradication of this disease (reviewed in reference Moss, B. Science 252:1662-1667, 1991). Vaccinia viruses have the advantages of low cost, heat stability and a simple method of administration. Attempts have been made to develop vaccinia virus vectors for the prevention of other diseases.

Vaccina virus is a member of the pox virus family of cytoplasmic DNA viruses. DNA recombination occurs during replication of pox viruses and this has been used to insert DNA into the viral genome. Recombinant vaccina virus expression vectors have been extensively described. These vectors can confer cellular immunity against a variety of foreign gene products and can protect against infectious diseases in several animal models. Recombinant vaccina viruses have been used in human clinical trials as well. Cooney et al immunized 35 healthy HIV seronegative males with a recombinant vaccinia virus expressing the gp160 envelope gene of HIV (Cooney, E.. The Lancet 337:567-572, 1991). Graham et al randomized 36 volunteers to receive either recombinant vaccinia virus containing the gp160 HIV envelope protein or control vaccinia virus (Graham, B.S. et al J. Infect. Dis. 166:244-252, 1992).

- 7 -

Phase I studies using recombinant vaccinia virus have begun in patients with metastatic melanoma using a recombinant virus expressing the p97 melanoma antigen (Estlin, C.D. et al Proc. Nat'l Acad. Sci. 85:1052-1056, 1988) and a trial to use recombinant vaccinia virus expressing the human carcinoembryonic antigen in patients with advanced colorectal carcinoma is about to begin (Schlom, J. personal communication). In these trials, vaccinia virus is administered by intradermal scarification and side effects have been minimal including local skin irritation, lymphadenopathy and transient flu-like symptoms.

Fowlpox viruses are members of the pox virus family (avipox virus genes). Fowlpox virus will only replicate in avian cells and cannot replicate in human cells. It is a cytoplasmic virus that does not integrate into the host genome but is capable of expression of a large number of recombinant genes in eukaryotic cells.

Recombinant fowlpox virus expressing rabies glycoprotein has been used to protect mice, cats and dogs against live rabies virus challenge. Immunization of chickens and turkeys with a recombinant fowlpox expressing the influenza HA antigen protected against a lethal challenge with influenza virus (Taylor, J. et al Vaccine 6:504-508, 1988). Canarypox virus, another member of the avipox genus similar to fowlpox, was safely administered subcutaneously to 25 normal human volunteers at doses up to 10⁸ infectious doses (Cadox, M. et al The Lancet 339:1429-1432, 1992). In a recent trial sponsored by the NIAID (Protocol 012A: A Phase I safety and immunogenicity trial of live recombinant canarypox-gp160 MN (ALVAC VCP125 HIV-1gp160MN0 in HIV-1 uninfected adults) patients received recombinant canarypox virus containing the HIV gp160 gene by intramuscular injection at doses up to 10⁸ pfu with little to no toxicity (personal communication, P. Fast, NIAID).

- 8 -

Fowlpox virus thus represents an attractive vehicle for immunization since it can stimulate both humoral and cellular immunity, it can be economically produced in high titer (10^9 pfu/ml) and yet its inability to productively infect human cells substantially increases the safety of its use, compared to replicating viruses such as vaccinia virus, especially in immunocompromised hosts.

Another considerable advantage of fowlpox virus is that there is apparently little or no cross-reactivity with vaccinia virus and thus previously vaccinated humans will not have pre-existing immune reactivity to fowlpox virus proteins.

SUMMARY OF THE INVENTION

The present invention is a recombinant virus comprising a viral genome or portion thereof, one or more nucleic acid sequences encoding one or more antigens of a disease causing agent and one or more nucleic acid sequences encoding one or more immunostimulatory molecules.

The present invention is also a composition comprising a recombinant virus comprising a viral genome or portion thereof and one or more nucleic acid sequences encoding one or more antigens of a disease causing agent and optionally an exogenous immunostimulatory molecule, chemotherapeutic drug, antibiotic, antifungal drug, antiviral drug, or combination thereof.

Another aspect of the present invention is a composition comprising a recombinant virus comprising a viral genome or portion thereof, one or more nucleic acid sequences encoding one or more antigens of a disease causing agent and one or more nucleic acid sequences encoding one or more immunostimulatory molecules and optionally an exogenous immunostimulatory molecule, chemotherapeutic drug, antibiotic, antifungal drug, antiviral drug, or combination thereof.

Another aspect of the present invention is to provide

- 9 -

0 a therapeutic composition and a method of treating or preventing a disease in a mammal comprising administering to the mammal an effective amount of a recombinant virus and optionally an exogenous immunostimulatory or immunomodulator, chemotherapeutic drug antibiotic, 5 antifungal drug, antiviral molecule, the amount effective in preventing or ameliorating the disease.

It is also an object of the present invention to provide a method of making an immune enhancing recombinant virus against a disease causing agent comprising inserting 10 a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one or more immunostimulatory molecules into the genome or portion thereof of a recombinant virus.

It is also an object of the present invention to provide an immune enhancing recombinant virus against a disease causing agent comprising an immune enhancing recombinant virus comprising a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one or more 20 immunostimulatory molecules.

It is also an object of the present invention to provide a vaccine against a disease causing agent comprising a recombinant virus containing a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one 25 or more immunostimulatory molecules. The vaccine of the present invention is able to prevent or inhibit infection or disease caused by the disease causing agent.

BRIEF DESCRIPTION OF THE DRAWINGS

30 These and other objects, feature and many of the attendant advantages of the invention will be better understood upon a reading of the detailed description of the invention.

Figure 1 shows β -Galactosidase production after BSC1 35 cell infection with various recombinant vaccinia virus

- 10 -

constructs after 24 hr (■) or 36 hr (□) incubation.

Figure 2 shows the primary response of mice at Day 6 after injection I.V. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (□); IL-2rVV (●); TNF α rVV (▼); IFN γ rVV(▼); VJS6 (O). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 3 shows the results from secondary cultures of mice at Day 6 after injection I.V. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (□); IL-2rVV (●); TNF α rVV (▼); IFN γ rVV(▼); VJS6 (O). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 4 shows the results from secondary cultures of mice at Day 14 after injection I.V. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (□); IL-2rVV (●); TNF α rVV (▼); IFN γ rVV(▼); VJS6 (O). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 5 shows the primary response of mice against vaccinia-infected CT26 tumor cells at Day 6 after injection S.C. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (●); IL-2rVV (□); TNF α rVV (▼); IFN γ rVV(▼); VJS6 (■). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 6 shows the titration of lytic units 30% from a primary response of mice against vaccinia-infected CT26 tumor cells at Day 6 after injection S.C. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (●); IL-2rVV (□); TNF α rVV (▼); IFN γ rVV(▼); VJS6 (■). Effector cells per well is plotted versus % specific ^{51}Cr release from CT26VAC target cells.

Figure 7 shows the results from secondary cultures of mice at Day 14 after injection S.C. with various recombinant vaccinia virus constructs as follows: GM-

- 11 -

CSFrVV (□); IL-2rVV (●); TNFrVV (▼); IFN γ rVV(▼); VJS6 (○). Effector: target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 8 shows active anti-tumor immunotherapy in mice using exogenous IL-2 together with recombinant vaccinia virus expressing a tumor associated antigen. Treatment groups included HBSS (—); rVV+BG (— -); rVV-BG + HD IL-2 (100,000 I.U., I.P., BID x 3 days) (—); rVV+BG + HD IL-2 (100,000 I.U., I.P., BID x 3 days) (- - - -); rVV + BG + LD IL-2 (15,000 I.U., BID x 3 days) (—). Survival time (days) is plotted versus proportion of surviving mice.

Figure 9 shows survival data in an active-tumor immunotherapy model in mice with an established non-transduced tumor, CT26. GM-CSFrVV (▲); IL-2rVV (■); TNFrVV (●); IFN γ rVV(◆); VJS6 (▼); HBSS (●). Days after tumor injection is plotted versus % surviving mice.

Figure 10 shows survival data in an active tumor immunotherapy model in mice with established β -gal expressing tumor, CT 26.C25. GM-CSFrVV (▲); IL-2rVV (■); TNFrVV (●); IFN γ rVV(◆); VJS6 (▼); HBSS (●). Days after tumor injection is plotted versus % surviving mice.

Figure 11 shows active treatment of established pulmonary metastases using various recombinant vaccinia constructs. The average number of pulmonary metastases is plotted versus recombinant vaccinia vector used for treatment. HB2m = Human Beta-2-microglobulin rVV, Ld = murine H-2L^d MCH Class I molecule rVV, NA = Neuramimidase rVV, MVA = Ankara - attenuated vaccinia virus, Kb = murine H-2K^b MCH Class I molecule rVV.

Figure 12 shows active treatment of established pulmonary metastases using various recombinant vaccinia constructs. The average number of pulmonary metastases is plotted versus recombinant vaccinia vector used for treatment. ICAM-1 = Intracellular adhesion molecule-1 rVV, D^d = murine H2-D^d MCH Class I molecule rVV, K^d =

- 12 -

murine H-2 K^d MHC Class I molecule rVV, L^d = H-2 L^d MHC Class I molecule rVV; K^b = H-2K^b MHC Class I molecule rVV; HLA-A2.1 = Human MHC Class I molecule rVV, hβ_{2m} = Human Beta-2-microglobulin rVV, NA = neuramimidase rVV, GM-CSF = granulocyte, monocyte colony stimulatory factor rVV, IFNγ = interferon gamma rVV, TNFα = tumor necrosis factor alpha rVV, MVA = Ankara - attenuated vaccinia virus, HBSS = Hank's Balanced Salt Solution.

Figure 13 shows a Western blot analysis of recombinant vaccinia virus expressed murine B7-1. BS-C-1 cells were infected with 1, v. MCB7-1; 2, v. MCMHA and extracts were prepared 20 hours post infection. A murine specific B7-1 hamster monoclonal antibody was used to detect expression of recombinant proteins. ¹²⁵I protein A (0.1 μCi/ml) and autoradiography were used to identify bound antibody. Protein sizes were estimated using ¹⁴C molecular weight markers (MW).

Figure 14 shows the results of non-irradiated BALB/c mice (5/group) which were injected intravenously with 5 x 10⁵ tumor cells of CT26.WT or CT26.C25 on day #0. On day #3 mice were immunized intravenously with different recombinant vaccinia viruses (10⁷ PFUs). Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. All mice injected with CT26.WT had 500 metastases (data not shown). This graph represents a compilation of data drawn from several separate experiments. The individual rVV were involved in the following number of experiments: v. IL2/β-gal -- five; v. MCB7-1/β-gal -- six; v. D^d/β-gal -- one; v. K^d/β-gal -- one, v. L^d/β-gal -- one, v. K^b/β-gal -- one, v. HLA.A2.1/β-gal -- one, v. mβ_{2m}/β-gal -- one, v. hβ_{2m}/β-gal -- one, v. GM-CSF/β-gal -- four, v. IFN-γ/β-gal -- four, v. TNF-α/β-gal -- four; v. JS6 -- greater than five, v. MVA/β-gal --- one.

Figure 15 shows the results of non-irradiated BALB/c

- 13 -

°
mice (10/group) which were injected intravenously with HBSS, v. MCB7-1/ β -gal, or v. MCMHA/ β -gal. Twenty-one days later mice were challenged intravenously with 5×10^5 tumor cells CT26.C25 or CT26.WT. Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. Duplicate experiments confirmed these results.

10 Figure 16 shows the results of non-irradiated BALB/c mice (10/group) which were injected with 10^5 or 10^7 PFUs of v. MCB7-1/ β -gal, v. MCMHA/ β -gal, or HBSS on day #0. Twenty-one days later a splenectomy was performed on all immunized mice. 2×10^7 splenocytes from designated groups of immunized mice were adoptively transferred to similar mice (5/group) injected intravenously three days earlier with 5×10^5 tumor cells of CT26.C25 or CT26.WT. Designated groups of mice were treated with IL-2 (100,000 IU i.p. BID x 3 days). Mice were randomized and euthanized nine days later. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. All mice inoculated with CT26.WT had large tumor burdens (data not shown). Duplicate experiments confirmed these results.

25 Figure 17 shows the results of non-irradiated BALB/c mice (5/group) which were injected intravenously with 5×10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #3 mice were immunized intravenously with 10^7 PFUs of v. MCB7-1/ β -gal, v. MCMHA/ β -gal, v. MCB7-1/NP or HBSS. On day# 3 - 6, designated groups of mice were treated with IL-2 (100,000 IU i.p. BID x 3 days). Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. All mice inoculated with CT26.WT had large tumor burdens (data not shown). Four separate experiments confirmed these results.

35 Figure 18 shows the results of non-irradiated BALB/c

- 14 -

0 mice (5/group) which were injected intravenously with 5×10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #6 mice were immunized intravenously with 10^7 PFUs of v. MCB7-1/ β -gal, v. MCMHA/ β -gal, v. MCB7-1/NP or HBSS. On
5 day# 6 - 9, designated groups of mice were treated with IL-2 (100,000 IU i.p. BID x 3 days). Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. All mice inoculated
10 with CT26.WT had large tumor burdens (data not shown). Duplicate experiments confirmed these results.

Figure 19 shows the results of non-irradiated BALB/c mice (10/group) which were injected intravenously with 5×10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #3 mice were immunized intravenously with 10^7 PFUs
15 of v. MCB7-1/ β -gal, v. MCMHA/ β -gal, v. MCB7-1/NP or HBSS. On day# 3 - 6, designated groups of mice were treated with IL-2 (100,000 IU i.p. BID x 3 days). All mice inoculated with CT26.WT were dead by day # 41 (data not
20 shown). Survival was followed daily and events recorded as deaths.

Figure 20 shows the results of non-irradiated BALB/c mice (5/group) which were injected intravenously with 5×10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #3 mice were immunized intravenously with 10^7 PFUs of v.
25 MCB7-1/ β -gal, v. MCB7-2/ β -gal, v. MCB7-1/B7-2/ β -gal, v. MCMHA/B7-2/ β -gal, v. MCMHA/ β -gal, v. MCB7-1/NP, or HBSS. On day# 3 - 6, designated groups of mice were treated with IL-2 (100,000 IU i.p. BID x 3 days). Mice were randomized, and euthanized on day #12. Lung were
30 harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. All mice inoculated with CT26.WT had large tumor burdens (data not shown). Duplicate experiments confirmed these results.

35 Figures 21a through 21f show a FACS analysis of splenocytes isolated from normal non-depleted (Figs. 21a

- 15 -

and 21b), T_{CD4+} -depleted (Figs. 21c and 21d), or T_{CD4+} -depleted (Figs. 21e and 21f) mice. Mice were given two i.v. injections of GK1.5 at 100 mg/ml or of empirically determined levels of 2.43 monoclonal antibodies 48 hours prior to receiving tumor challenge, and again 6 days later. Upper left hand panel in each square displays percent CD8+ cells, upper right panel shows percent CD4+CD8+ cells, lower right shows percent CD4+, and lower left shows scatter for all cells. Figure 21a, no depletion Using fluorescein isothiocyanate-labeled anti-CD4 and phycoerythrin labeled anti-CD8 antibodies, FACS analysis was performed 1 day prior to immunization, and again at day 7 to verify depletion.

Figure 21g shows the mean number of pulmonary metastases of CD4- and CD8- immuno depleted mice vaccinated with v.B7-1/ β -gal. Prior to tumor challenge non-irradiated BALB/c mice (5/group) were injected with anti-CD4 and anti-CD8 monoclonal antibodies (GK1.5 (anti-CD4) and 2.43 (anti-CD8). On day#0 mice were injected intravenously with 5×10^5 CT26.C25 tumor cells. On day #3 mice were immunized intravenously with 10^7 PFUs of v. MCB7-1/ β -gal, v. MCMHA/ β -gal, v. MCB7-1/NP or HBSS. On day #6, the mice were again injected with anti-CD4 and anti-CD8 monoclonal antibodies. Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. FACS analysis was performed 1 day prior to immunization, and again at day 7 to verify depletion. Only data from v.B7-1/ β -gal vaccination groups is shown. Duplicate experiments confirmed these results.

Figures 22a and 22b show active immunotherapy is enhanced when exogenous rIL-2 and rVV are given in concert. BALB/c mice (five per group) were challenged i.v. with 5×10^5 CT26.WT (Fig. 22a) or CT26.CL25 tumor cells (Fig. 22b). After 3 days they received a single i.v. injection of medium alone (HBSS) or medium containing

- 16 -

5 x 10⁶ PFU of different TK rVV either expressing (VJS6) or not expressing (V69) β -gal. Two different regimens of rIL-2 administration were started 12 h after rVV injection: high dose (HD, 100,000 Cetus U, twice a day, i.p. for 3 days) or low dose (LD, 15,000 Cetus U, twice a day, i.p. for 5 days). Mice were checked twice a day for survival.

Figures 23a and 23b show that exogenous rIL-2 enhances the function of rFPV. BALB/c mice (five per group) were inoculated i.v. with 5 x 10⁵ CT26.WT (Fig. 23a) or CT26.CL25 tumor cells (Fig. 23b). On day 3 after tumor injection, they received a single i.v. injection of the following viruses: no virus (HBSS alone), 10⁷ PFU of FPV.bg40k (rVPV), or FPV wild-type (FPVwt). rIL-2 (100,000 Cetus U, twice a day) was administered i.p. starting 12 h after FPV injection and continued for 3 days. Mice were checked twice a day for survival.

Figure 24 shows exogenous rIL-2 plus rVV is therapeutic in the more advanced 6-day tumor model. BALB/c, mice (five per group) were inoculated i.v., with 10⁵CT26.CL25 tumor cells. Six days after tumor injection, they received the same treatments described in Figures 22a and 22b with the exception that only the highest dose of rIL-2 was administered. Mice were checked daily for survival. No prolongation of survival was obtained by the various treatments in mice bearing 6-day-old pulmonary metastases of CT26.WT tumor (data not shown).

Figures 25a and 25b show a drVV expressing IL-2, but not GM-CSF, IFN- γ , or TNF- α , significantly reduces the number of pulmonary metastases in a 3-day model. Five BALB/c mice per group were injected i.v. with 5 x 10⁵ tumor cells of either CT26.WT or CT26.CL25 cell lines. Three days later they received a single i.v. injection of HBSS alone (none) or containing 5 x 10⁶PFU/mouse of different rVV, as indicated. On day 12 post tumor challenge, lungs were harvested and pulmonary nodules were

- 17 -

enumerated in a blind fashion. An independent repeat of this experiment gave identical results.

Figures 26a and 26b show the function of a drVV expressing IL-2 is further enhanced when additional exogenous IL-2 is provided. BALB/c mice (five per group) were challenged i.v. with 5×10^5 CT26.WT (Fig. 26a) or CT26.CL25 tumor cells (Fig. 26b). After 3 days they received a single i.v. injection of plain medium (HBSS) or medium containing 5×10^6 PFU of rVV encoding β -gal alone (VJS6) or together with IL-2 (IL-2 rVV). Twelve hours after rVV, 100,000 rIL-2U were inoculated, according to the regimen described in Figs. 22a and 22b. Mice were checked twice a day for survival.

Figure 27 shows that expression of IL-2 by drVV enhances the antivaccinia CTL response. Two BALB/c mice were immunized with 5×10^6 PFU/mouse of different rVV. After 6 days the spleens were aseptically removed, mixed together, and tested directly in a 6-h ^{51}Cr release assay against CT26.WT tumor cell line, either infected (CT26 vaccinia) or noninfected (CT26.WT) during the isotope labeling with more than 10:1 moi of crude 19 VV preparation. Spontaneous release of target cells never exceeded 20%. E:T cell ratio was 100:1 and then 1:3 dilutions. Lytic units 30% (L.U. 30%) indicate the number of effector cells required to obtain 30% lysis of 10,000 target cells. L.U. 30% were normalized for the total number of cells recovered for each spleen and expressed as total L.U./spleen.

Figure 28 shows that the presence of tumor cells specifically enhance the CTL response elicited by IL-2 rVV in a dose-dependent manner. BALB/c mice were injected with HBSS alone or with varying doses of CT26.WT or CT26.CL25 as specified. After 3 days, mice were immunized with 5×10^6 PFU/mouse of either VJS6 or IL-2 rVV. On day 9 after tumor challenge (day 6 after vaccination) the primary cytotoxic response was evaluated in a 6-h ^{51}Cr

- 18 -

release assay against CT26.WT, CT26.WT pulsed with the β -gal L^d-restricted peptide (CT26T + peptide), CT26.CL25, and the irrelevant target cells E22 as shown. The effects of escalating doses of tumor on the generation of the primary cytotoxic response is shown for the immunization with IL-2rVV in nontumor-bearing mice (Δ), or in mice bearing 5×10^4 CT26.CL25 (Δ), 1×10^5 CT26.CL25 (\bullet), 5×10^5 CT26.CL25 (O), or the highest dose, 5×10^5 of the parental (non- β -gal-expressing) CT26.WT cell line (\square). An additional control is the non-IL-2-expressing VJS6 virus injected in mice bearing 5×10^5 CT26.CL25 (\blacksquare).

Figure 29 shows the effects of recombinant IL-10 on the therapeutic effectiveness of immunization with the vaccinia virus, VJS6 + IL-1 (\bullet); VJS6 alone (X).

Figure 30 shows the IL-10 adjuvant therapy on the therapeutic effectiveness of immunization with various concentrations of recombinant vaccinia virus encoding the model tumor antigen.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a novel recombinant virus expressing an antigen(s) from a disease causing agent and an immunostimulatory molecule(s). The novel recombinant virus is capable of eliciting or upregulating an immune response in a mammal to T-dependent antigens for the purpose of preventing or treating a disease. The novel recombinant virus of the present invention is particularly important in upregulating cell-mediated immunity.

Cell-mediated immunity is crucial to resistant to diseases caused by cancer and pathogenic microorganism, particularly viruses and other intracellular microorganisms.

The recombinant virus has incorporated into its genome or portion thereof a gene encoding an antigen from a disease causing agent and one or more genes encoding one or more immunostimulatory molecules. A host cell infected

- 19 -

with the recombinant virus expresses both the antigen(s) from a disease causing agent and expresses the immunostimulatory molecule(s). The antigen may be expressed at the cell surface of the infected host cell. The immunostimulatory molecule may be expressed at the cell surface or may be actively secreted by the host cell.

The expression of both the antigen and the immunostimulatory molecule provides the necessary MHC restricted peptide to specific T cells and the appropriate signal to the T cell to aid in antigen recognition and proliferation or clonal expansion of antigen specific T cells. The overall result is an upregulation of the immune system. In a preferred embodiment the upregulation of the immune response is an increase in antigen specific cytotoxic lymphocytes which are able to kill or inhibit the growth of a disease causing agent or a cell infected with a disease causing agent.

In one embodiment, the recombinant virus comprises the virus genome or portions thereof, the nucleic acid sequence encoding an antigen from a pathogenic microorganism and one or more nucleic acid sequences encoding one or more immunostimulatory molecules.

In another embodiment, the recombinant virus comprises the virus genome or portions thereof, the nucleic acid sequence encoding a tumor associated antigen, and one or more nucleic acid sequences encoding one or more immunostimulatory molecules.

In one embodiment the recombinant viruses have been constructed and co-express model tumor antigens together with cytokines (TNF- α , IFN- γ , GM-CSF, IL-10 and IL-2), restriction elements (class 1 α -chains and β_2m), and co-stimulatory and accessory molecules (B7-1, B7-2 and ICAM-1 and the like) alone and in a variety of combinations. Simultaneous production of an immunostimulatory molecule and the model TAA at the site of virus replication/infection (in any case, the site of TAA

- 20 -

production) enhances the generation of specific effectors. Dependent upon the specific immunostimulatory molecules, different mechanisms might be responsible for the enhanced immunogenicity: augmentation of help signal (IL-2), recruitment of professional APC (GM-CSF), increase in CTL frequency (IL-2), effect on antigen processing pathway and MHC expression (IFN γ and TNF α) and the like. The co-expression of a model antigen together with at least one immunostimulatory molecule is effective in an active immunotherapy model.

The present invention also encompasses a recombinant virus comprising the virus genome or portion thereof, the nucleic acid sequence encoding the antigen of interest and more than one nucleic acid sequences encoding more than one immunostimulatory molecule for the added benefit of upregulating an immune response against the antigen.

In some cases it may be beneficial to make a recombinant virus comprising more than one antigen of interest for the purpose of having a multivalent vaccine. For example, the recombinant virus may comprise the virus genome or portions thereof, the nucleic acid sequence encoding GP120 (from HIV), the nucleic acid sequence encoding Hep B surface antigen and one or more immunostimulatory molecules.

In one embodiment, the recombinant virus comprises the vaccinia virus genome or portions thereof, the nucleic acid sequence encoding MART-1 and the nucleic acid sequence encoding the immunostimulatory molecule, B 7.1 alone or in combination with the nucleic acid sequence encoding the immunostimulatory molecule, B7.2.

In another embodiment, the recombinant virus comprises the fowlpox virus genome or portions thereof, the nucleic acid sequence encoding MART-1, the nucleic acid sequence encoding MAGE-1, and the nucleic acid sequence encoding the immunostimulatory molecule, IL-2, alone or in combination with the nucleic acid sequence

- 21 -

encoding the immunostimulatory molecule, B7.1. In a specific embodiment the recombinant virus is McB7-1, McB71-1/NP, McB7-1 β -gal, McB7-1/ova, McB7-1/PIA, McB7-1/B7-2, McMHA, McMHA/NP, McMHA/ β -gal, McMHA/ova, McMHA/PIA, McMHA/B7-2, McB7-2, McB7-2/ β -gal, IL-2(β -gal)rVV, GM-CSF(β -gal)rVV, IFN γ (β -gal)rVV, IL-10(β -gal)rVV, and TNF α (β -gal)rVV.

The insertion of costimulatory molecules and/or cytokine genes in recombinant vaccinia virus (rVV genome containing the model TAA) is beneficial in treatment of established metastases.

The present invention encompassed a composition comprising a recombinant virus containing a viral genome or portion thereof, one or more nucleic acid sequences encoding one or more antigens from one or more disease causing agents and one or more nucleic acid sequences encoding one or more immunostimulatory molecules. The compositions of the present invention may also comprise an exogenous immunostimulatory molecule or combinations of immunostimulatory molecules and/or may comprise a chemotherapeutic drug, antibiotic, antifungal drug, antiviral drug and the like and combinations thereof.

In one embodiment, the composition contains a recombinant virus which has incorporated into its genome or portion thereof a gene encoding a tumor associated antigen and exogenous IL-2. In another embodiment, the composition contains a recombinant virus which as incorporated into its genome or portion thereof a gene encoding a tumor associated antigen in combination with exogenous IL-10.

Virus Vectors

Virus that may be used in the present invention are those in which a portion of the genome can be deleted to introduce new genes without destroying infectivity of the virus. The virus vector of the present invention is a nonpathogenic virus. In one embodiment the virus vector

- 22 -

has a tropism for a specific cell type in the mammal. In another embodiment, the virus vector of the present invention is able to infect professional antigen presenting cells such as dendritic cells and macrophages. In yet another embodiment of the present invention, the virus vector is able to infect any cell in the mammal. The virus vector may also infect tumor cells.

The virus of the present invention include but is not limited to Poxvirus such as vaccinia virus, fowlpox virus and a highly attenuated vaccinia virus (Ankara or (MVA)), retrovirus, adenovirus, baculovirus and the like.

The vaccinia virus genome is known in the art. It is composed of a HIND F13L region, TK region, and an HA region. The recombinant vaccinia virus has been used in the art to incorporate an exogenous gene for expression of the exogenous gene product (Perkus et al. Science 229:981-984, 1985; Kaufman et al. Int. J. Cancer 48:900-907, 1991; Moss Science 252:1662, 1991).

A general strategy for construction of vaccinia virus expression vectors have been described (Smith and Moss Bio Techniques Nov/Dec, p. 306-312, 1984; U.S. Patent No. 4,738,846). Briefly, the first step in formation of recombinant viruses expressing heterologous DNA is the construction of a chimeric gene containing a vaccinia promoter fused to the protein coding sequences of the foreign gene. The chimeric gene is assembled in a plasmid vector and engineered so that the transcriptional start site of the promoter is positioned close to the translational initiation codon of the foreign gene. Importantly, additional ATG triplets between the transcriptional and translational start sites should be eliminated so that fusion polypeptides or incorrect reading frames are avoided. Generally, the promoters are included in DNA fragments 200-300 base pairs in length, but smaller fragments have also proved functional. After their assembly in plasmid vectors, the chimeric genes are

- 23 -

°
inserted into the virus genome by homologous recombination
in vivo. This method is necessary since the great size of
vaccinia DNA makes *in vitro* construction of recombinant
molecules impractical. To facilitate homologous
5 recombination, the chimeric gene is first flanked by
vaccinia DNA taken from a non-essential region of the
virus genome. The resulting plasmid, called a
recombination vector, is transfected into vaccinia virus-
infected cells, whereupon homologous recombination results
10 in site-specific insertion of the chimeric gene into the
virus genome. Recombinant genomes are replicated and
packaged into infectious progeny virus within the infected
cells. The nature of the flanking DNA is important since
this determines the site of insertion. Only nonessential
15 regions can be used and several of these have now been
identified. A commonly used locus is the vaccinia TK gene
since recombinant viruses are consequently TK.

A gene encoding an antigen of a disease causing agent
may be incorporated into the HIND F13L region or
alternatively incorporated into the TK region of
20 recombinant vaccinia virus vector. Likewise, a gene
encoding an immunostimulatory molecule may be incorporated
into the HIND F13L region or the TK region of recombinant
vaccinia virus vector.

25 Sutter and Moss (Proc. Nat'l. Acad. Sci U.S.A.
89:10847-10851, 1992) and Sutter et al. (Virology 1994)
disclose the construction and use as a vector, the non-
replicating recombinant Ankara virus (MVA, modified
vaccinia Ankara) which may be used as a viral vector in
the present invention.

30 Baxby and Paoletti (Vaccine 10:8-9, 1992) disclose
the construction and use as a vector, of the non-
replicating poxvirus, including canarypox virus, fowlpox
virus and other avian species which may be used as a viral
vector in the present invention.

35 Expression vectors suitable for use in the present

- 24 -

invention comprise at least one expression control element operationally linked to the nucleic acid sequence. The expression control elements are inserted in the vector to control and regulate the expression of the nucleic acid sequence. Examples of expression control elements includes, but is not limited to, lac system, operator and promoter regions of phage lambda, yeast promoters and promoters derived from polyoma, adenovirus, retrovirus or SV40. Additional preferred or required operational elements include, but are not limited to, leader sequence, termination codons, polyadenylation signals and any other sequences necessary or preferred for the appropriate transcription and subsequent translation of the nucleic acid sequence in the host system. It will be understood by one skilled in the art the correct combination of required or preferred expression control elements will depend on the host system chosen. It will further be understood that the expression vector should contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the host system. Examples of such elements include, but are not limited to, origins of replication and selectable markers. It will further be understood by one skilled in the art that such vectors are easily constructed using conventional methods (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, New York) or commercially available.

Disease Causing Agents

The recombinant virus of the present invention is effective in treating or preventing disease caused by disease causing agents or a disease state. Each disease causing agent or disease state has associated with it an antigen or immunodominant epitope on the antigen which is crucial in immune recognition and ultimate elimination or control of the disease causing agent in a mammal,

- 25 -

sometimes referred to in the art as a protective antigen. The mammalian immune system must come in contact with the antigen or immunodominant epitope on the antigen in order to mount a humoral and/or cellular immune response against the associated disease causing agent.

The recombinant virus of the present invention comprises the one or more nucleic acid sequences encoding one or more isolated antigens or immunodominant epitopes on the antigens and one or more nucleic acid sequences encoding one or more immunostimulatory molecules for the purpose of enhancing immune response against the disease causing agent.

Such disease causing agents include but are not limited to cancer and pathogenic microorganisms or mammals. Mammals include but are not limited to humans, primates, rats, mice, guinea pigs, rabbits, horses, cows, sheep, pigs, goats and the like. Cancers of mammals which may be treated using the recombinant virus of the present invention include but are not limited to melanoma, metastases, adenocarcinoma, thymoma, lymphoma, sarcoma, lung cancer, liver cancer, colon cancer, non-Hodgkins lymphoma, Hodgkins lymphoma, leukemias, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer and the like.

The term melanoma includes, but is not limited to, melanomas, metastatic melanomas, melanomas derived from either melanocytes or melanocytes related nevus cells, melanocarcinomas, melanoepitheliomas, melanosarcomas, melanoma in situ, superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, invasive melanoma or familial atypical mole and melanoma (FAM-M) syndrome. Such melanomas in mammals may be caused by, chromosomal abnormalities, degenerative growth and developmental disorders, mitogenic agents, ultraviolet radiation (UV), viral infections,

- 26 -

inappropriate tissue expression of a gene, alterations in expression of a gene, and presentation on a cell, or carcinogenic agents.

The aforementioned cancers can be assessed or treated by methods described in the present application. In the case of cancer, a gene encoding an antigen associated with the cancer is incorporated into the recombinant virus genome or portion thereof along with a gene encoding one or more immunostimulatory molecules. The antigen associated with the cancer may be expressed on the surface of a cancer cell or may be an internal antigen. In one embodiment the antigen associated with the cancer is a tumor associated antigen (TAA) or portion thereof.

Examples of TAA that may be used in the present invention include but are not limited to melanoma TAAs which include but are not limited to MART-1 (Kawakami et al. J. Exp. Med. 180:347-352, 1994), MAGE-1, MAGE-3, GP-100, (Kawakami et al. Proc. Nat'l. Acad. Sci. U.S.A. 91:6458-6462, 1994), CEA, TRP-1, P-15, and tyrosinase (Brichard et al. J. Exp. Med. 178:489, 1993) and the like.

The nucleotide sequence of the MAGE-3 gene is disclosed in Gaugler et al. J. Exp. Med. 179:921-930, 1994. MAGE-3 is expressed on many tumors of several types, such as melanoma, head and neck squamous cell carcinomas, lung carcinoma and breast carcinoma but not in normal tissues except for testes. The approximately 1.6 Kilobase (kb) cDNA of MART-1 was cloned into a vector and the resulting plasmid, deposited with the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD 20852 USA on April 14, 1994, and given ATCC Deposit Number 75738. The cloning of MART-1 is disclosed in Kawakami et al J. Exp. Med. 180:347-352, 1994 and Serial No. 08/231,565 filed April 22, 1994. The full length MART-1 nucleic acid sequence can be isolated from the pCRII plasmid by digestion with HINDII and XhoI restriction enzymes. This 1.6kb nucleic acid sequence or

- 27 -

portions thereof can then be incorporated into the genome of the recombinant viruses described herein along with an immunostimulatory gene or genes.

In another embodiment the TAAs are CA-19-A (pancreatic cancer), CA-125 (ovarian cancer), PSA (prostate cancer), erb-2 (breast cancer, CA-171A) and the like (Boon et al. Ann. Rev. Immunol 12:337, 1994).

The present invention is in no way limited to the genes encoding the above listed TAAs. Other TAAs may be identified, isolated and cloned by methods known in the art such as those disclosed in U.S. Patent No. 4,514,506.

Genes encoding an antigen of a disease causing agent in which the agent is a pathogenic microorganism in mammals and include viruses such as HIV (GP-120, p17, GP-160 antigens), influenza (NP, HA antigen), herpes simplex (HSVdD antigen), human papilloma virus, equine encephalitis virus, hepatitis (Hep B Surface Antigen) feline leukemia virus, canine distemper, rabies virus, and the like. Pathogenic bacteria include but are not limited to Chlamydia, Mycobacteria, Legionella and the like. Pathogenic protozoans include but are not limited to malaria, Babesia, Schistosoma, Toxoplasma, Toxocara canis, and the like. Pathogenic yeast include Aspergillus, invasive Candida, and the like. In a preferred embodiment the pathogenic microorganism is an intracellular organism.

Immunostimulatory Molecules: Costimulation/Accessory Molecules and Cytokines

The gene from costimulation/accessory molecule and/or gene encoding an a cytokine in combination with a gene encoding an antigen from a disease causing agent is incorporated into the genome of a recombinant virus. Examples of costimulation molecules include but are not limited to B7-1, B7-2, ICAM-1, ICAM-2, LFA-1, LFA-3, CD72 and the like. Examples of cytokines encompassed by the present invention include but are not limited to IL-2, IL-

- 28 -

1, IL-3 through IL-9, IL-11, IL-13 through IL-15, G-CSF, M-CSF, GM-CSF, TNF α , IFN α , IFN γ , IL-10, IL-12, Regulated upon activation, normal T expressed and presumably secreted cytokine (RANTES), and the like. Examples of chemokines encompassed by the present invention include
5 but are not limited to CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MBSA, MIP-1 α , MIP-1B and the like.

IL-2 construct

The IL-2 gene of the present invention was made made as disclosed by Taniguchi et al (Structure and expression of a cloned cDNA for human interleukin-2 Nature 302:305, 1983).

In one embodiment the entire IL-2 gene as disclosed in Taniguchi et al is incorporated into the TK gene sequence of vaccinia virus.

15 The promotor sequence for the IL-2 construct of the present invention is made up of the P synthetic late promotor as disclosed in Davidson et al Nucleic Acid Research 18 (No. 14):4285-4286, 1991.

Also encompassed in the present invention is a
20 chimeric gene containing a pox virus promotor region linked to the coding segment of one or more foreign genes encoding an antigen(s) from a disease causing agent and the coding segment of one or more foreign genes encoding an immunostimulatory molecule(s). The chimeric genes then
25 incorporated into the pox virus genome by homologous recombination in cells that have transfected with a plasmid vector containing the chimeric gene and infected with the pox virus.

In a one embodiment the IL-2 construct of the present
30 invention comprises the recombinant vaccinia containing the IL-2 gene and the P synthetic late promotor and an antigen in the TK region from a disease causing agent in the F13L region of the vaccinia virus genome promotor.

B7 Construct

35 Co-stimulatory molecules of the B7 family (namely

- 29 -

°
B7.1, B7.2, and possibly B7.3) represent a more recently discovered, but important group of molecules. B7.1 and B7.2 are both member of the Ig gene superfamily. These molecules are present on macrophages, dendritic cells, monocytes, i.e., antigen presenting cells (APCs). If a lymphocyte encounters an antigen alone, with co-stimulation by B7.1, it will respond with either anergy, or apoptosis (programmed cell death); if the co-stimulatory signal is provided it will respond with clonal expansion against the target antigen. No significant amplification of the immune response against a given antigen occurs without co-stimulation (June et al. (Immunology Today 15:321-331, 1994); Chen et al. (Immunology Today 14:483-486); Townsend et al. (Science 259:368-370)). Freeman et al. (J. Immunol. 143:2714-2722, 1989) report cloning and sequencing of B7.1 gene. Azuma et al. (Nature 366:76-79, 1993) report cloning and sequencing B7.2 gene.

In one embodiment the B7.1 gene was inserted into the Hind F13L region of the vaccinia virus, with the β -gal placed in the TK region. The construct for B7.2 and B7.1/B7.2 in conjunction with a tumor antigen are prepared in the same fashion as B7.1.

In another embodiment the B7 gene is inserted into the TK region of vaccinia virus and the gene encoding β -gal inserted in the Hind F13L region of the vaccinia virus.

The IFN γ construct, TNF α construct, GM-CSF construct and ICAM-1 construct were constructed as disclosed in Davidson et al Nucleic Acid Research 18 (No. 14):4285-4286, 1991.

The present invention also encompasses methods of treatment or prevention of a disease caused by the disease causing agents disclosed here.

In the method of treatment, the administration of the recombinant virus of the invention may be for either

- 30 -

° "prophylactic" or "therapeutic" purpose. When provided prophylactically, the recombinant virus of the present invention is provided in advance of any symptom. The prophylactic administration of the recombinant virus serves to prevent or ameliorate any subsequent infection or disease. When provided therapeutically, the recombinant virus is provided at (or shortly after) the onset of a symptom of infection or disease. Thus the present invention may be provided either prior to the anticipated exposure to a disease causing agent or after the initiation of the infection or disease.

The genetic definition of tumor-specific antigens allows for the development of targeted antigen-specific vaccines for cancer therapy. Insertion of a tumor antigen gene in the genome of viruses in combination with a immunostimulatory molecule is a powerful system to elicit a specific immune response in terms of prevention in patient with an increased risk of cancer development (preventive immunization), prevention of disease recurrence after primary surgery (anti-metastatic vaccination), or as a tool to expand the number of CTL in vivo, thus improving their effectiveness in eradication of diffuse tumors (treatment of established disease). Finally, recombinant viruses of the present invention can elicit an immune response in patient that is enhanced ex vivo prior to being transferred back to the tumor bearer (adoptive immunotherapy).

The term "unit dose" as it pertains to the inoculum refers to physically discrete units suitable as unitary dosages for mammals, each unit containing a predetermined a quantity of recombinant virus calculated to produce the desired immunogenic effect in association with the required diluent. The specifications for the novel unit dose of an inoculum of this invention are dictated by and are dependent upon the unique characteristics of the recombinant virus and the particular immunologic effect to

- 31 -

be achieved.

The inoculum is typically prepared as a solution in tolerable (acceptable) diluent such as saline, phosphate-buffered saline or other physiologically tolerable diluent and the like to form an aqueous pharmaceutical composition.

The route of inoculation may be intravenous (I.V.), intramuscular (I.M.), subcutaneous (S.C.), intradermal (I.D.) and the like, which results in eliciting a protective response against the disease causing agent. The dose is administered at least once. Subsequent doses may be administered as indicated.

In providing a mammal with the recombinant virus of the present invention, preferably a human, the dosage of administered recombinant virus will vary depending upon such factors as the mammal's age, weight, height, sex, general medical condition, previous medical history, disease progression, tumor burden and the like.

In general, it is desirable to provide the recipient with a dosage of recombinant virus in the range of from about 10^5 to about 10^{10} plaque forming units/mg mammal, although a lower or higher dose may be administered.

The recombinant viral vector can be introduced into a mammal either prior to any evidence of cancers such as melanoma or to mediate regression of the disease in a mammal afflicted with a cancer such as melanoma. Examples of methods for administering the viral vector into mammals include, but are not limited to, exposure of cells to the recombinant virus *ex vivo*, or injection of the recombinant virus into the affected tissue or intravenous S.C., I.D. or I.M. administration of the virus. Alternatively the recombinant viral vector or combination of recombinant viral vectors may be administered locally by direct injection into the cancerous lesion or topical application in a pharmaceutically acceptable carrier. The quantity of recombinant viral vector, carrying the nucleic acid

- 32 -

sequence of one or more TAAs to be administered is based on the titer of virus particles. A preferred range of the immunogen to be administered is 10^5 to 10^{10} virus particles per mammal, preferably a human.

After immunization the efficacy of the vaccine can be assessed by production of antibodies or immune cells that recognize the antigen, as assessed by specific lytic activity or specific cytokine production or by tumor regression. One skilled in the art would know the conventional methods to assess the aforementioned parameters. If the mammal to be immunized is already afflicted with cancer or metastatic cancer the vaccine can be administered in conjunction with other therapeutic treatments.

In one method of treatment, autologous cytotoxic lymphocytes or tumor infiltrating lymphocytes may be removed from the patient with cancer as disclosed in U.S. Patent No. 5,126,132 and U.S. Patent No. 4,690,915. The lymphocytes are grown in culture and antigen specific lymphocytes expanded by culturing in the presence of the recombinant virus of the present invention. The antigen specific lymphocytes are then reinfused back into the patient.

The present invention also encompasses combination therapy. By combination therapy is meant that the recombinant virus containing one or more genes encoding one or more antigens associated with one or more disease agents and one or more genes encoding one or more immunostimulatory molecules is administered to the patient in combination with other exogenous immunomodulators or immunostimulatory molecules, chemotherapeutic drugs, antibiotics, antifungal drugs, antiviral drugs and the like alone or in combination thereof. In one embodiment the combination therapy includes a recombinant virus and exogenous IL2. In another embodiment the combination therapy includes a recombinant virus encoding one or more

- 33 -

exogenous antigens and exogenous IL-10. Examples of other exogenously added agents include exogenous IL-2, IL-6, IL-12, GM-CSF, interferon, IL-10, tumor necrosis factor, RANTES (Promega, G5661), cyclophosphamide, and cisplatin, gancyclovir, amphotericin B and the like.

This invention further comprises an antibody or antibodies elicited by immunization with the recombinant virus of the present invention. The antibody has specificity for and reacts or binds with the antigen of interest. In this embodiment of the invention the antibodies are monoclonal or polyclonal in origin.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules or those portions of an immunoglobulin molecule that contain the antigen binding site, including those portions of immunoglobulin molecules known in the art as F(ab), F(ab'); F(ab)₂, and F(v). Polyclonal or monoclonal antibodies may be produced by methods known in the art. (Kohler and Milstein (1975) Nature 256, 495-497; Campbell "Monoclonal Antibody Technology, the Production and Characterization of Rodent and Human Hybridomas" in Burdon et al. (eds.) (1985) "Laboratory Techniques in Biochemistry and Molecular Biology," Volume 13, Elsevier Science Publishers, Amsterdam). The antibodies or antigen binding fragments may also be produced by genetic engineering. The technology for expression of both heavy and light chain genes in E. coli is the subject of the PCT patent applications: publication number WO 901443, WO 901443 and WO 9014424 and in Huse et al. (1989) Science 246:1275-1281.

In one embodiment the antibodies of this invention are used in immunoassays to detect the novel antigen of interest in biological samples.

In one embodiment, the MART-1 antibodies of this invention generated by immunization with recombinant

- 34 -

°
vaccinia virus expressing MART-1 and B7.1 are used to
assess the presence of the MART-1 antigen from a tissue
biopsy of a mammal afflicted with melanoma using
immunocytochemistry. Such assessment of the delineation
of the MART-1 antigen in a diseased tissue can be used to
5 prognose the progression of the disease in a mammal
afflicted with the disease or the efficacy of
immunotherapy. Conventional methods for
immunohistochemistry are described in (Harlow and Lane
(eds) (1988) In "Antibodies A Laboratory Manual", Cold
10 Spinning Harbor Press, Cold Spring Harbor, New York;
Ausbel et al. (eds) (1987). In Current Protocols In
Molecular Biology, John Wiley and Sons (New York, New
York).

15 In another embodiment the antibodies of the present
invention are used for immunotherapy. The antibodies of
the present invention may be used in adoptive
immunotherapy.

20 In providing a patient with the antibodies or antigen
binding fragments to a recipient mammal, preferably a
human, the dosage of administered antibodies or antigen
binding fragments will vary depending upon such factors as
the mammal's age, weight, height, sex, general medical
condition, previous medical condition and the like.

25 In general, it is desirable to provide the recipient
with a dosage of antibodies or antigen-binding fragments
which is in the range of from about 1 mg/Kg to about 10
mg/Kg body weight of the mammal, although a lower or
higher dose may be administered.

30 The antibodies or antigen-binding fragments of the
present invention are intended to be provided to the
recipient subject in an amount sufficient to prevent,
lessen or attenuate the severity, extent or duration of
the disease or infection.

- 35 -

Example 1

Construction and Characterization of Recombinant
Vaccinia Viruses Expressing Murine B7
(mB7) and Additional Foreign Proteins

Various methods for inserting foreign genes into a
5 vaccinia virus expression vector are known in the art
(Smith, G.L. et al, Vaccinia Virus Expression Vectors:
Construction, Properties and Applications Bio Techniques
Nov/Dec:306-311, 1984; Flexner, C. et al. Expression of
Human Interleukin-1 by live recombinant vaccinia virus.
10 Vaccines 87, Cold Spring, Harbor Lab., pp 380-383; U.S.
Patent No. 4,738,846). These as well as other techniques
may be employed to construct the recombinant virus of the
present invention. Recombinant vaccinia viruses
expressing mB7 and additional extrinsic proteins were
15 constructed. The mB7 and additional foreign genes were
inserted (by homologous recombination) into the vaccinia
VP37 (Hind III F) and TK (Hind III J) genes. For control
viruses, the mB7 gene was replaced by the measles HA (mHA)
gene.

20 Construction and Characterization of Vaccinia Recombinant
Viruses Expressing mB7-1, mB7-2 and mHA.

The construction of initial recombinants expressing
the above genes employed the plaque formation selection
system in which the foreign gene is directed into the
25 vaccinia F13L (VP37) loci. Briefly, the foreign gene is
cloned into a vaccinia transfer plasmid (pRB21) adjacent
to the synthetic early late promoter (E/L) and flanked by
DNA homologous to the F13L region in vaccinia. A full-
length F13L gene (under control of its authentic promoter)
is also situated within the flanking regions. The mB7-1,
30 mB7-2 and mHA were individually cloned adjacent to E/L
within pRB21. A virus that has a plaque deficient
phenotype (due to deletion of the F13L plaque forming
gene) was used for transfection and the proceeding
35 homologous recombination of plasmid DNA. Transfections

- 36 -

° were carried out according to standard published procedures. Recombinant viruses were readily identified according to their plaque positive phenotype, in contrast to the plaque deficient non-recombinants.

5 Partially plaque purified recombinants from each transfection were consequently characterized using antibodies specific for the individual foreign protein expressed. This was carried out by direct immuno-staining of plaques on unfixed monolayers of BS-C-1 cells. As cells were unfixed this also indicated that the target
10 proteins were expressed on the cell surface. For the majority of studies rat anti-mB7-1 and B7-2 MAbs (Pharmagen) were used to identify surface expression of these proteins. The vaccinia recombinants were plaque purified at least 4 times on BS-C-1 cells (African Green
15 monkey kidney cell line American Type Culture Collection (ATCC Accession No. CCL 26) followed by plaquing on STO cells embryonic mouse fibroblast, Accession No. ATCC/CRL 1503 in the presence of 6-thioguanine (TG) (Sigma Chemical Co.) which selects against unstable single cross-over
20 recombinants. Recombinants were again plaque purified on BS-C-1 cells at least once before stocks were grown in HeLa Spinner cells. The virus was semi-purified by ultracentrifugation through a 36% sucrose cushion. Aliquots were prepared and stored at 80°C. A single
25 aliquot from each batch was thawed, sonicated and titred on BSC-1 cells. Infected monolayers were immunostained to determine continued expression of foreign proteins.

The B7-1 recombinant (v.MCB7.1) was further characterized in a FACS analysis to illustrate surface
30 expression and its ability to bind CTLA4-Ig (B7-1 T-cell receptor) and by Western blot analysis using the anti-B7-1 antibody.

The B7-1 recombinant has been shown to be attenuated compared to wild-type WR, in a mouse LD⁵⁰ assay.

35 **Construction and Characterization of Vaccinia Recombinants**

- 37 -

°

mB7-1 , mB7-2 or mHa Co-expressing Additional Genes

Additional genes were inserted into the TK region of the above recombinants. The following is a list of recombinants constructed and characterized (the first part of the code indicates the gene situated in the F13L loci under control of the E/L promoter and latter abbreviation denotes genes in the TK region):

	<u>Code</u>	<u>Genes Expressed and Promoters</u>
10	McB7-1	mB7-1, P. E/L
	McB71-1/NP	mB7-1, P. E/L Influenza NP, P7.5K
15	McB7-1 β -gal	mB7.1, P.E/L B-gal. P.7.5K
	McB7-1/ova	mB7-1, P.E/L Ovalbumin, P11.K B-gal, 11k
20	McB7-1/P1A	mB7-1, P.E/L Murine P1A, P7.5K β -gal. P.11k
	McB7-1/B7-2	mB7-1, P.E/L B-gal, P.11k
25	McMHA	Measle Hemagglutinin, P.E./L
	McMHA/NP McMHA/ β -gal	
30	McMHA/ova McMHA/P1A	TK inserted, details as above
	McMHA/B7-2	mB7-2, 7.5K B-gal. P.11k
35	McB7-2	B7-2. P.E./L

- 38 -

McB7-2/ β -gal B7-2, P.E./L
 B-gal. P.11k

5 A recombinant vaccinia virus containing the genes for B7.1 and the tumor associated antigen, β -gal was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA under Accession No. VR2485 according to terms of the Budapest Treaty. A recombinant vaccinia virus containing the genes for IL-2 and β -gal was
10 deposited with ATCC under Accession No. VR2486.

Sucrose cushion purified stocks of all characterized recombinant viruses have been prepared.

15 The above method is also employed to construct other recombinant virus using the other previously mentioned viruses such as fowlpox virus in combination with other foreign antigens and immunostimulatory molecules.

Example 2

Recombinant Fowlpox Virus Expressing A TAA, LacZ and B7.1

20 A recombinant fowlpox virus (rFPV), which is replication incompetent in mammalian cells, is constructed that express the model TAA, lacZ encoding β -gal, and the immunostimulatory molecule, B7.1 under the influence of the 40K vaccinia virus early/late promotor.

25 The POXVAC-TC (Schering Corp.) strain of FPV is used. FPV is propagated on primary chick embryo dermal cultures (Jenkins, S. et al. AIDS Res. Hum. Retroviruses, 7:991, 1991). Foreign sequences are inserted into FPV by homologous recombination as previously described (Jenkins
30 et al. 1991). Recombinant fowlpox contains the *E. coli* lacZ gene and the B7.1 gene under the control of the vaccinia virus 40K promotor (designed H6 in Rosel, J. et al. J. Virol. 60:436, 1986), inserted into the BAM HI J region of the FPV genome.